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Eudesmane-Type Sesquiterpenoid and Guaianolides from *Kandelia candel* in a Screening Program for Compounds to Overcome TRAIL Resistance

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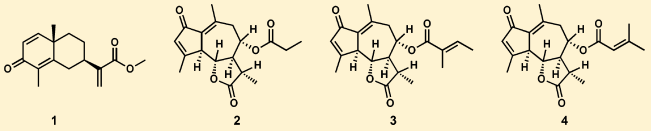
Supporting Information

ABSTRACT: In a screening program for natural products that can overcome TRAIL resistance, a new eudesmane-type sesquiterpenoid (**1**), three new guaianolides, mehirugins A−C (**2**−**4**), and two known guaianolides (**5** and **6**) were isolated from a MeOH extract of *Kandelia candel* leaves. Compounds **1** and **3**−**6** in combination with TRAIL showed cytotoxic activity in sensitizing TRAIL-resistant human gastric adenocarcinoma cells.



Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has emerged as a promising agent with the ability to kill tumor cells selectively. TRAIL-induced apoptosis initiated by the death-receptor pathway involves death-receptor (DR4 and DR5) engagement, death-inducing signaling complex formation, proteolytic activation of caspase-8, and, consequently, activation of caspase-3. Proteolytic caspase-8 further activates Bid (BH3 interacting domain death agonist), which, in turn, translocates to the mitochondria and activates the mitochondrial pathway. However, a considerable number of cancer cells, especially highly malignant tumors, are resistant to TRAIL. Though reduced levels of cell surface DR4 and DR5, enhanced levels of decoy receptors (DcRs), and an increased level of apoptosis inihibitors [cFLIP (cellular FLICE-like inhibitory protein) and BCL-2 (B-cell CLL/lymphoma 2) family] have been described as the mechanisms of TRAIL resistance, a general consensus for resistance to TRAIL has not yet been identified.[1] Identifying compounds that can overcome TRAIL resistance has thus become an important strategy for discovering anticancer drugs. During a search for bioactive natural products with tumor-selective apoptosis-inducing activity,[2,3] we examined the MeOH extracts of medicinal plants collected in Bangladesh and Thailand. We previously reported several active compounds that influence TRAIL resistance, isolated from *Amoora cucullata*,[4] *Sida acuta*,[5] and *Combretum quadrangulare*.[6] In addition, we suggested that some of these compounds overcome TRAIL resistance by upregulating DR5 expression. Moreover, in the primary screening of a library of extracts, the MeOH extract of *Kandelia candel* (Rhizophoraceae) leaves was found to reduce TRAIL resistance. Plants of the genus *Kandelia* (Japanese name, Mehirugi) are evergreen mangrove trees, and *K. candel* is a species common to South and Southeast Asia. To date, several proanthocyanidin dimers have been isolated from the bark of this plant.[7] In this paper, we describe the activity-guided isolation and structural elucidation of four new sesquiterpenoids (**1**−**4**) along with two known compounds (**5** and **6**) from *K. candel* and their effects on TRAIL resistance.

RESULTS AND DISCUSSION

The MeOH extract of *K. candel* leaves, which exhibited activity to overcome TRAIL resistance (42% more inhibition than the agent alone) at 100 μg/mL, was subjected to diaion HP20 column chromatography, and a fraction eluted with MeOH was partitioned between *n*-hexane, EtOAc, *n*-BuOH, and H_2O. The activity was found to be strong in the *n*-hexane- and EtOAc-soluble fractions. Activity-guided fractionation of the *n*-hexane-soluble fractions by silica gel column chromatography and preparative HPLC yielded compounds **1**−**6**. Compounds **5** and **6** were identified as the known sesquiterpenoids notoserolide B[8] and matricarin,[9] respectively, by comparison of their spectroscopic data with literature data.

Compound **1** was shown to have the molecular formula $C_{16}H_{20}O_3$ on the basis of HRESIMS data (*m*/*z* 283.1312, calcd for $C_{16}H_{20}O_3Na$, $[M + Na]^+$, Δ +0.2 mmu), which indicated seven degrees of hydrogen deficiency. The IR spectrum exhibited absorption bands at ν_{max} 1716 and 1635 cm^{-1} due to carbonyl groups. The ^1H NMR spectrum (Table 1) showed two methyls [δ_H 1.88 (3H, br s), 1.22 (3H, s)], one methoxy [δ_H 3.74 (3H, s)], two coupled sp^2 methines [δ_H 6.73 (1H, d, J = 10.0 Hz) and 6.21 (1H, d, J = 10.0 Hz)], and one sp^2 methylene [δ_H 5.63 (1H, br s) and 6.23 (1H, s)]. The ^{13}C NMR spectrum showed 16 carbons, comprising two carbonyl

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Chart 1

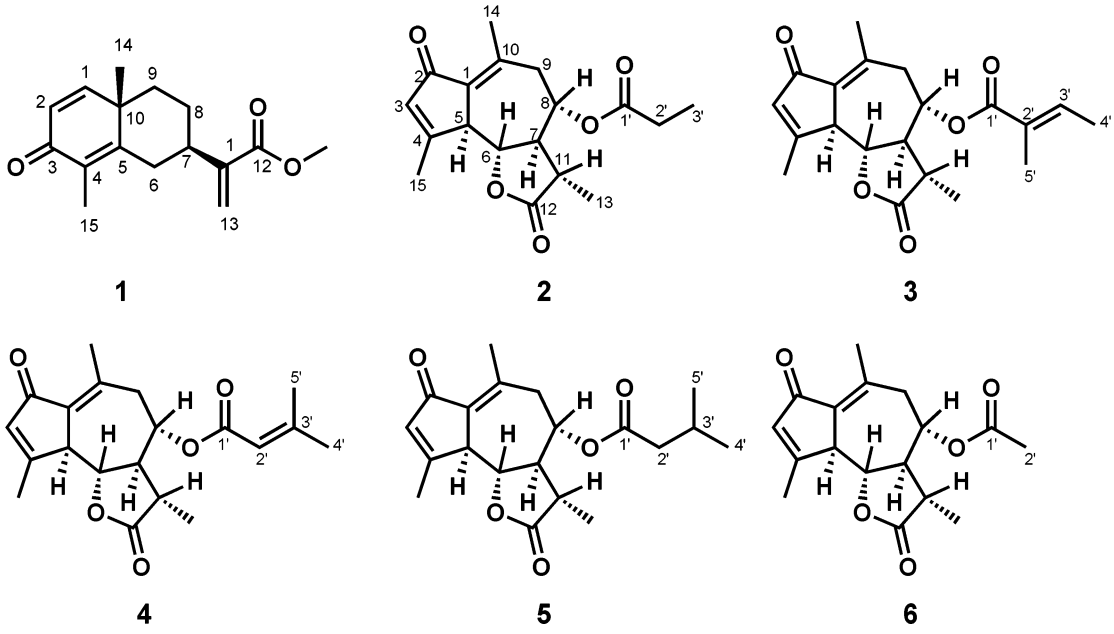

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for 1 in $CDCl_3$

	δ_C		δ_H (J in Hz)
1	156.5	CH	6.73 d (10.0)
2	126.2	CH	6.21 d (10.0)
3	186.5	qC	
4	129.6	qC	
5	158.9	qC	
6	33.1	CH_2	2.17 br t (12.3)
			2.89 br dd (3.5, 12.3)
7	40.9	CH	2.47 br tt (3.5, 12.3)
8	26.4	CH_2	1.72−1.75 m
9	37.7	CH_2	1.38 dt (5.5, 12.6)
			1.82 dt (3.5, 12.6)
10	40.1	qC	
11	143.8	qC	
12	167.1	qC	
13	123.8	CH_2	5.63 br s, 6.23 s
14	23.5	CH_3	1.22 s
15	10.5	CH_3	1.88 br s
1′	51.9	CH_3	3.74 s

carbons (δ_C 186.5, 167.1), two sp^2 methines (δ_C 156.5, 126.2), three sp^2 quaternary carbons (δ_C 158.9, 143.8, 129.6), one sp^2 methylene (δ_C 123.8), one methoxy carbon (δ_C 51.9), one sp^3 methine (δ_C 40.9), one sp^3 quaternary carbon (δ_C 40.1), three sp^3 methylenes (δ_C 37.7, 33.1, 26.4), and two methyls (δ_C 23.5, 10.5) by DEPT and HMQC analyses. Five of the seven degrees of hydrogen deficiency were accounted for by two carbonyls, three double bonds, and the presence of two rings.

The $^{1}H-^{1}H$ COSY spectrum indicated partial structures (Figure 1) from H-1 to H-2 (partial structure **a**) and H_2-6 to H_2-9 (partial structure **b**). The HMBC cross-peaks from H-1 and H_3-15 to C-3 (δ_C 186.5), H-2 and H_3-15 to C-4 (δ_C 129.6), H-1 and H_3-15 to C-5 (δ_C 158.9), and H-2 to C-10 (δ_C 40.1) indicated the presence of a 2,5-cyclohexadienone moiety including partial structure **a** (ring **A**) with a methyl group at C-4. The connectivity of partial structure **b** and ring **A** through

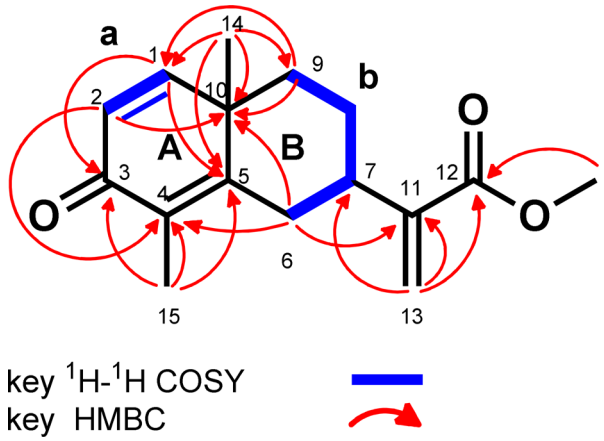

Figure 1. Key $^{1}H-^{1}H$ COSY and HMBC of **1**.

a quaternary carbon (C-10) bearing a methyl group was indicated by the HMBC cross-peaks from H_2-6 to C-4 and C-10, H_2-9 to C-1 and C-10, and H_3-14 to C-1, C-5, C-9, and C-10. The presence of a 2-substituted acrylic acid methyl ester moiety at C-7 was suggested by the HMBC cross-peaks from H_2-6 to C-11, H_2-13 to C-7, C-11, and C-12, and OMe to C-12. Comparison of ^{1}H NMR data with those of 3-oxoeudesma-1,4,11(13)-trien-12-oic acid[10] (**7**) supported that the structure of **1** was as above. The axial orientation of H-7 was deduced from the large coupling constants ($J_{6\beta,7}$ = 12.3 and $J_{7,8\beta}$ = 12.3 Hz). Alkaline hydrolysis afforded a product whose physical data were identical to those of **7** (^{1}H NMR, MS, and $[\alpha]_D$),[10] and, thus, **1** was identified as the methyl ester of **7**.

Mehirugin A (**2**) was obtained as a colorless solid, and its molecular formula $C_{18}H_{22}O_5$, with two less carbon and two less hydrogen atoms than notoserolide B[8] (**5**), was determined from the $[M + Na]^+$ peak at m/z 369.1321 (calcd for $C_{18}H_{22}O_5Na$ $[M + Na]^+$, Δ −4.4 mmu) in the HRESIMS. A comparison of ^{1}H NMR spectra indicated that **2** had a similar structure to **5**. The senecioyl (3-methylbut-2-enoyl) group [δ_H 1.92 (3H, d, J = 1.1 Hz), 2.19 (3H, d, J = 1.1 Hz), and 5.63 (1H, septet, J =

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Table 2. ^{1}H NMR Spectroscopic Data for 2−6 in $CDCl_3$

	2	3	4	5	6
	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
3	6.16 quint (1.1)	6.17 quint (1.3)	6.16 quint (1.3)	6.16 quint (1.1)	6.19 br quint (1.3)
5	3.37 br d (10.0)	3.38 br d (10.0)	3.37 br d (10.7)	3.37 br d (10.5)	3.39 br d (10.6)
6	3.67 t (10.0)	3.72 t (10.0)	3.69 t (10.7)	3.70 t (10.5)	3.72 t (10.6)
7	2.35−2.40 m	2.35−2.40 m	2.32 dt (11.9, 10.7)	2.32 dt (11.9, 10.5)	2.34 dt (11.9, 10.6)
8	4.79 dt (2.1, 10.8)	4.91 dt (1.9, 10.7)	4.80 dt (1.9, 10.7)	4.81 dt (2.1, 10.5)	4.83 dt (1.9, 10.6)
9	2.35 dd (2.1, 13.5)	2.35 dd (1.9, 13.6)	2.33 dd (1.9, 13.5)	2.35 dd (2.1, 13.6)	2.36 dd (1.9, 13.6)
	2.66 dd (10.8, 13.5)	2.70 dd (10.7, 13.6)	2.67 dd (10.7, 13.5)	2.66 dd (10.5, 13.6)	2.71 dd (10.6, 13.6)
11	2.44 dq (11.9, 6.8)	2.49 dq (11.9, 6.8)	2.46 dq (11.9, 6.8)	2.45 dq (11.9, 6.8)	2.49 dq (11.9, 6.8)
13	1.32 d (6.8)	1.28 d (6.8)	1.32 d (6.8)	1.29 d (6.8)	1.33 d (6.8)
14	2.42 br s	2.43 s	2.43 s	2.43 s	2.43 s
15	2.28 br s	2.28 br s	2.28 br s	2.28 br s	2.30 br s
2′	2.35 q (7.5)		2.20 dd (15.0, 6.9)	5.63 sep (1.1)	2.12 s
	2.36 q (7.5)		2.23 dd (15.0, 7.3)		
3′	1.16 t (7.5)	6.90 br q (7.0)	2.08−2.18 m		
4′		1.82 br d (7.0)	0.98 d (6.6)	2.19 d (1.1)	
5′		1.84 br s	0.98 d (6.6)	1.92 d (1.1)	

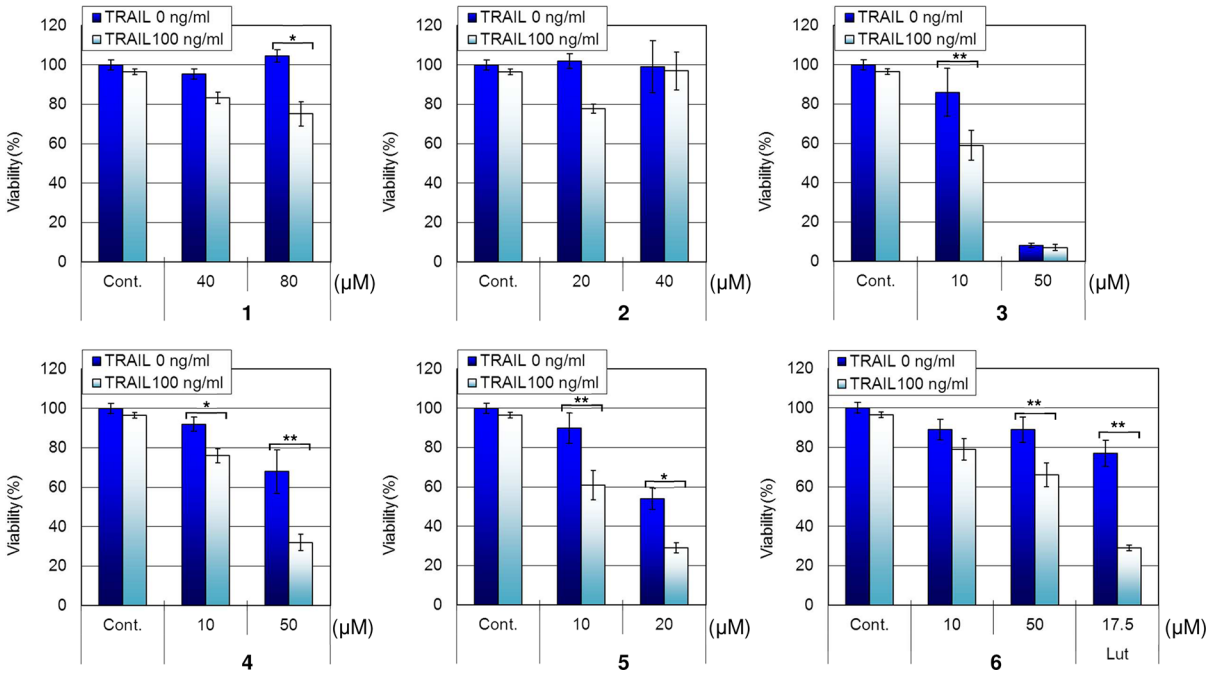

Figure 2. Effects of compounds **1**−**6**, luteolin (positive control: Lut), and DMSO (negative control: con) in the presence or absence of TRAIL on the viability of AGS cells. The cells were seeded in a 96-well culture plate (6×10^3 cells per well) for 24 h and then treated with the indicated concentrations of the compounds and TRAIL (100 ng/mL) for 24 h. Bars represent the mean ± SD (n = 3). *p < 0.05; **p < 0.01, Tukey's test.

1.1 Hz)] in **5** was replaced by a propanoyl group [δ_H 2.35 (1H, q, J = 7.5 Hz), 2.36 (1H, q, J = 7.5 Hz), and 1.16 (1H, t, J = 7.5 Hz)]. The coupling constants for **2** ($J_{5,6}$ = 10.0 Hz, $J_{6,7}$ = 10.0 Hz, $J_{7,8}$ = 10.8 Hz, and $J_{7,11}$ = 11.9 Hz) closely resembled those for **5** and 8-*O*-methylsenecioylaustricin[11] ($J_{5,6}$ = 10.5 Hz, $J_{6,7}$ = 10.5 Hz, $J_{7,8}$ = 10.5 Hz, and $J_{7,11}$ = 11.9 Hz), which have 5H-α, 6H-β, 7H-α, 8H-β, and 11H-β orientations. J values were significantly different from those of daucoguaianolactone A[12] ($J_{5,6}$ = 11.2 Hz, $J_{6,7}$ = 9.9 Hz, $J_{7,8}$ = 11.1 Hz), which have 5H-β, 6H-α, 7H-α, and 8H-β orientations. From these observations, **2** has 5H-α, 6H-β, 7H-α, 8H-β, and 11H-β orientations.

Mehirugin B (**3**) had the molecular formula $C_{20}H_{24}O_5$, the same as that of **5**. Its ^{1}H and ^{13}C NMR spectra were similar to those of **5**. The senecioyl group observed in **5** was replaced by a tigloyl group [δ_H 1.82 (3H, br d, J = 7.0 Hz)/δ_C 14.5, 1.84 (3H, br s)/δ_C 12.0, and 6.90 (1H, br q, J = 7.0 Hz)/δ_C 139.0]. A comparison of the ^{1}H NMR data with those for **3** and 8α-tigloyloxydehydroleucodin[13] in the side chain moiety supported the presence of the tigloyl moiety.

Mehirugin C (**4**) had the molecular formula $C_{20}H_{26}O_5$, with two more hydrogen atoms than **5**. The ^{1}H NMR spectra of **4** also closely resembled those of **5**, with a difference in the signals of the side chain moiety. The senecioyl group in **5** was replaced by an isovaleroyl group [δ_H 0.98 (6H, d, J = 6.6 Hz), 2.08−2.18 (1H, m), 2.20 (1H, dd, J = 15.0, 6.9 Hz), 2.23 (1H, dd, J = 15.0, 7.3 Hz)], and the presence of the isovaleroyl group was supported by comparison of ^{1}H and ^{13}C NMR data with those for daucoguaianolactone A,[12] having the same side chain moiety.

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The similarity of the coupling constant values as shown in Table 2 revealed that **2**–**6** had the same configurations, and the large coupling constant values (10.0–11.9 H) for $J_{5,6}$, $J_{6,7}$, $J_{7,8}$, and $J_{7,11}$ revealed that H-5/H-6, H-6/H-7, H-7/H-8, and H-7/H-11 are all in *trans*-positions. The ECD spectra for **2**–**6** all showed similar curves exhibiting negative Cotton effects at around 260 nm, indicating that **2**–**6** have the same absolute configurations, although the absolute configurations of **5** and **6** had not yet been defined.[8,9]

The isolated compounds (**1**–**6**) were tested for activity to overcome TRAIL resistance in AGS cells. As shown in Figure 2, treatment with 100 ng/mL TRAIL for 24 h resulted in only a slight decrease in cell viability (3%), while luteolin[14] (Lut) at 17.5 μM, which was used as a positive control, produced about 48% stronger inhibition when administered in combination with TRAIL than TRAIL alone. Combined use of **5** (10 μM) and **3** (10 μM) with TRAIL produced a 29% and 27% greater decrease, respectively, in cell viability than treatment with compound alone (without TRAIL). Combined use of **1** (80 μM), **4** (50 μM), and **6** (50 μM) with TRAIL induced 29%, 36%, and 23% more inhibition of cell viability than treatment with each compound alone (without TRAIL), showing moderate TRAIL-resistance-overcoming activity.

Although TRAIL can induce selective apoptosis in tumor cells, studies have shown that a number of cancer cells are resistant to TRAIL, especially highly malignant tumors such as pancreatic cancer, neuroblastomas, and malignant melanomas.[1] Understanding the mechanisms of such resistance and developing strategies are important for the successful use of TRAIL in cancer therapy. Combined treatment with chemotherapeutic agents, including natural products, can overcome such resistance and enhance the therapeutic potential of TRAIL; therefore, a natural product that overcomes TRAIL resistance would be a new tool for investigating cancer cells.[15] In this paper, we reported for the first time the cytotoxic activity of eudesmane and guaiane sesquiterpenoid derivatives in the presence of TRAIL against TRAIL-resistant AGS cells, thereby suggesting their possible use in combination with TRAIL against human gastric adenocarcinoma.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured using the ATR (attenuated total reflection) method on a JASCO FT-IR 230 spectrophotometer. CD spectra were measured on a JASCO J-720WI spectropolarimeter. NMR spectra were recorded on JEOL JNM-AS00 and JEOL JNM-ECP600 spectrometers with a deuterated solvent, the chemical shifts of which were used as an internal standard. HRESIMS were obtained on a JEOL JMS-T100LP spectrometer. Column chromatography was performed using Diaion HP20 (Mitsubishi Chemical Co. Tokyo, Japan) and silica gel PSQ100B (Fuji Silysia Chemical Ltd., Kasugai, Japan). Preparative HPLC was performed using YMC-Pack ODS-AM and YMC-Pack Pro C18 (YMC Co., Ltd., Kyoto, Japan). The ratios of solvent are described as a mixture by v/v.

Plant Material. The leaves of *K. candel* were collected in Bangladesh in November 2008 and were taxonomically identified by Prof. A. K. Fazlul Huq, Forestry and Wood Technology Discipline, Khulna University, Bangladesh. A voucher specimen (KKB61) was deposited in our laboratory.

Extraction and Isolation. Air-dried and ground leaves of *K. candel* (113 g) were subjected to extraction with MeOH for 2 days at room temperature followed by homogenization and filtration, and then evaporation and vacuum desiccation to obtain a crude extract (8.1 g). The extract was chromatographed on Diaion HP-20 (6.3 × 25 cm; particle size 250–850 mm) to remove the chlorophyll. The chlorophyll-free fraction (6.0 g) was suspended in 10% aqueous MeOH (200 mL) and partitioned between *n*-hexane, EtOAc, and *n*-BuOH (200 mL × 3) to obtain the corresponding extracts. The *n*-hexane extract (443 mg) was subjected to silica gel PSQ100B column chromatography (1.8 × 42 cm) using the *n*-hexane/EtOAc solvent system with increasing polarity to afford compound **1** (36.8 mg) in the fraction eluted with *n*-hexane/EtOAc (4:1), together with fractions 1B to 1H. Fraction 1D (20.6 mg) was subjected to preparative HPLC [YMC-Pack ODS-AM 1.0 × 25 cm; MeOH/H_2O (2:1); flow rate: 2.0 mL/min; RI and UV detection at 254 nm] to give **2** (1.1 mg, t_R 14 min). Fraction 1C (16.3 mg) was subjected to preparative HPLC [YMC-Pack Pro C18 1.0 × 25 cm; MeOH/H_2O (2:1); flow rate: 1.5 mL/min; RI and UV detection at 254 nm] to give **3** (0.1 mg, t_R 28 min), **4** (3.6 mg, t_R 40 min), and **5** (0.9 mg, t_R 36 min). Fraction 1E (45.0 mg) was subjected to preparative HPLC [YMC-Pack ODS-AM 1.0 × 25 cm; MeOH/H_2O (2:1); flow rate: 1.0 mL/min; RI and UV detection at 254 nm] to afford **6** (5.5 mg, t_R 23 min).

Compound 1: yellow oil; $[\alpha]_D^{23}$ −54 (*c* 0.4, $CHCl_3$); UV (MeOH) λ_{max} 239 nm (log ε 2.9); IR (ATR) ν_{max} 1716 and 1635 cm^{-1}; ^1H and ^{13}C NMR (Table 1); HRESIMS *m*/*z* 283.1312 $[M + Na]^+$ (calcd for $C_{16}H_{20}O_3Na$, 283.1310).

Mehirugin A (2): colorless solid; $[\alpha]_D^{23}$ +3.4 (*c* 0.4, $CHCl_3$); UV (MeOH) λ_{max} 254 nm (log ε 2.8); IR (ATR) ν_{max} 1779, 1734, 1686, 1638, and 1617 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{263}$ −1.4; ^1H NMR (Table 2); HRESIMS *m*/*z* 341.1321 $[M + Na]^+$ (calcd for $C_{18}H_{22}O_5Na$, 341.1364).

Mehirugin B (3): colorless solid; $[\alpha]_D^{23}$ +64 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 253 nm (log ε 3.40), 220 nm (3.37); IR (ATR) ν_{max} 1778, 1687, 1641, and 1617 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{268}$ −4.9; ^1H and ^{13}C NMR (Tables 2 and 3); HRESIMS *m*/*z* 367.1485 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_5Na$, 367.1521).

Table 3. ^{13}C NMR Spectroscopic Data for 3 and 5 in $CDCl_3$

	3		5	
	δ_C		δ_C	
1	133.0	qC	133.0	qC
2	195.2	qC	195.6	qC
3	135.8	CH	135.8	CH
4	169.6	qC	169.6	qC
5	51.4	CH	51.5	CH
6	81.1	CH	81.1	CH
7	59.2	CH	59.2	CH
8	70.0	CH	69.0	CH
9	44.7	CH_2	44.8	CH_2
10	145.4	qC	145.4	qC
11	40.6	CH	40.9	CH
12	176.7	qC	176.9	qC
13	14.9	CH_3	14.9	CH_3
14	21.2	CH_3	21.4	CH_3
15	19.9	CH_3	19.9	CH_3
1′	166.4	qC	165.0	qC
2′	128.0	qC	114.9	CH
3′	139.0	CH	159.8	qC
4′	14.5	CH_3	20.4	CH_3
5′	12.0	CH_3	27.6	CH_3

Mehirugin C (4): colorless solid; $[\alpha]_D^{23}$ +21 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 255 nm (log ε 3.4); IR (ATR) ν_{max} 1773, 1733, 1684, 1646, and 1617 cm^{-1}; CD (MeOH) $\Delta\varepsilon_{264}$ −0.5; ^1H NMR (Table 2); HRESIMS *m*/*z* 369.1632 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_5Na$, 369.1677).

Compound 5: CD (MeOH) $\Delta\varepsilon_{263}$ −2.1 (not previously reported).

Compound 6: CD (MeOH) $\Delta\varepsilon_{259}$ −2.1 (not previously reported).

Alkaline Hydrolysis of 1. A solution of **1** (2.6 mg) in MeOH/H_2O (9:1) (0.88 mL) was treated with 1 N NaOH (0.88 mmol) at

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room temperature for 2 h. The reaction mixture was poured into H_2O and EtOAc and extracted with EtOAc. The organic layer was subjected to silica gel column chromatography eluted with *n*-hexane/EtOAc (3:2) to give **7**.

Cell Cultures. AGS cells were derived from the Institute of Development, Aging and Cancer, Tohoku University, and cultured in RPMI-1640 medium (Wako) with 10% fetal bovine serum (FBS). All cultures were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air. Luteolin (purity ≥98%, Sigma Aldrich) was used as a positive control.

Activity Assay. Effects on TRAIL resistance were assessed by a comparison of cell viability in the presence and absence of TRAIL using TRAIL-resistant human gastric adenocarcinoma (AGS) cell lines.[16] The AGS cells were seeded in a 96-well culture plate (6×10^3 cells per well) in 200 μL of RPMI medium containing 10% FBS. The cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Test samples at different concentrations with or without TRAIL (100 ng/mL) were added to each well. After 24 h incubation, the cells were washed with PBS, and 200 μL of PBS containing fluorescein diacetate[17] (10 μg/mL) was added to each well. The plates were incubated at 37 °C for 1 h, and fluorescence at 538 nm with excitation at 485 nm was measured using Fluoroskan Ascent (Thermo Scientific). The purity of all compounds tested was greater than 95% on the basis of TLC and NMR analysis. Values are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of the new compounds are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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